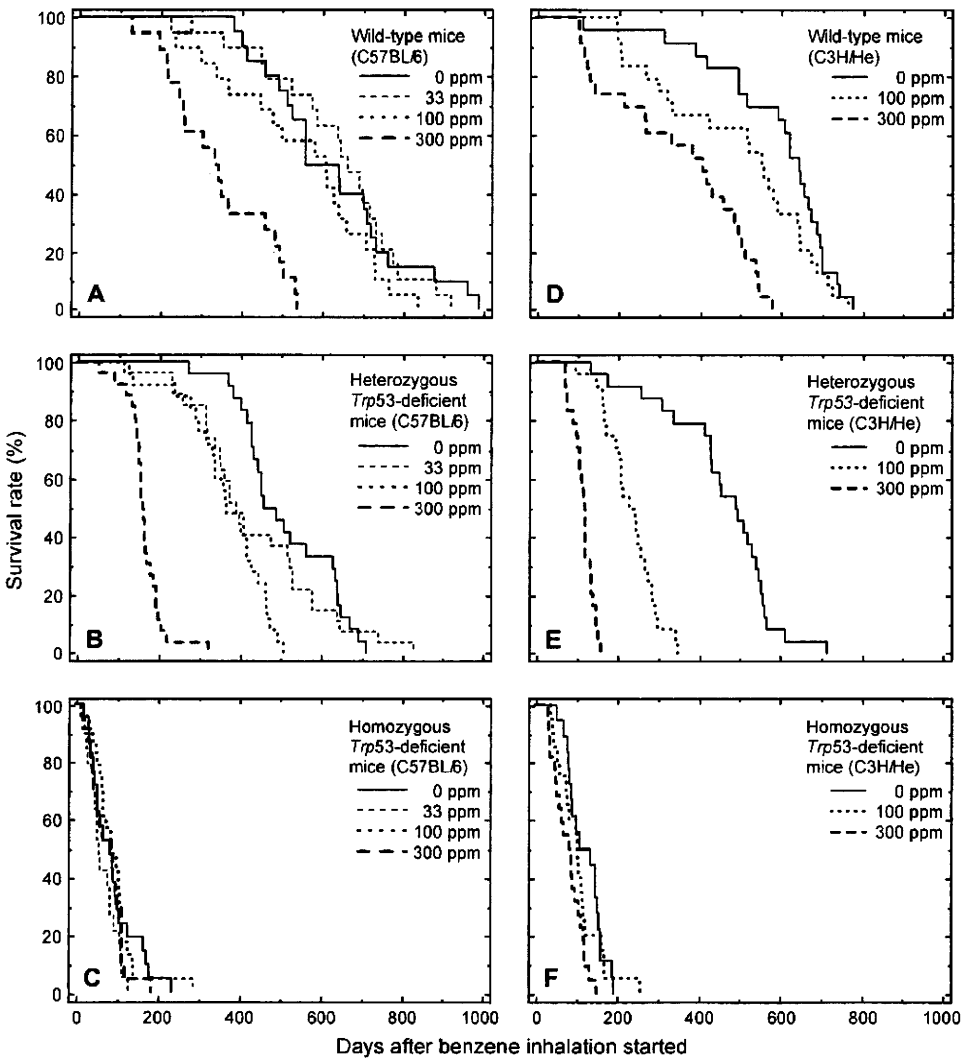

FIG. 1. Survival rates (%) are plotted on the vertical axis and survival time (days) after benzene inhalation on the horizontal axis, using (A)–(C) for C57BL/6 mice and (D)–(F) for C3H/He mice, with (A) and (D) for wild-type mice, (B) and (E) for heterozygous *Trp53*-deficient mice, and (C) and (F) for homozygous *Trp53*-deficient mice. Bold dotted lines for 300-ppm exposure group, regular dotted line for 100-ppm exposure group, fine dotted lines for 33-ppm exposure group, and solid line for sham exposure control. Statistical significance by log-rank test: (A) 0 versus 300 ppm, $p = 4.7 \times 10^{-06}$; 33 versus 300 ppm, $p = 8.3 \times 10^{-06}$; 100 versus 300 ppm, $p = 4.9 \times 10^{-04}$; (B) 0 versus 300 ppm, $p = 1.4 \times 10^{-10}$; 33 versus 300 ppm, $p = 4.8 \times 10^{-10}$; 100 versus 300 ppm, $p = 2.2 \times 10^{-08}$; 0 versus 100, $p = 1.9 \times 10^{-04}$; 33 versus 100 ppm, $p = 3.5 \times 10^{-02}$; (C) no significant difference between groups; (D) 0 versus 300 ppm, $p = 5.6 \times 10^{-06}$; 100 versus 300 ppm, $p = 1.0 \times 10^{-03}$; (E) 0 versus 300 ppm, $p = 5.8 \times 10^{-06}$; 100 versus 300 ppm, $p = 4.0 \times 10^{-09}$; 0 versus 100 ppm, $p = 1.1 \times 10^{-07}$; and (F) 0 versus 300 ppm, $p = 4.4 \times 10^{-03}$.

Cumulative Deaths due to HPNs

Benzene exposure in wild-type mice. The cumulative incidences of HPNs in each wild-type experimental group are shown in Figure 2A (C57BL/6) and Figure 2D (C3H/He). In C57BL/6 mice, the wild-type group exposed to 300 ppm showed a gradual increase in cumulative incidence of HPNs to 55.6% by day 532. In C3H/He mice, groups exposed to 100 and 300 ppm showed somewhat lower but similar increases in HPNs to 25.0% by 554 days and 30.4% by 431 days, respectively, as seen in Figure 2A (C57BL/6) and Figure 2D (C3H/He). With the exception of the 300-ppm exposure group of wild-type C57BL/6 mice, the incidence and onset of HPNs did not exceed 21.0% during lifetime observation (21.0% for the 33-ppm group and 15.8% for the 100-ppm groups). The maximum incidences of HPNs in the wild-type sham control group were 10.0% by 492 days in C57BL/6 mice and 8.7% by 742 days in C3H/He mice.

The first question in the present study concerned threshold-like equivocal incidence of HPNs at low-dose benzene exposure. In this regard, only the C57BL/6, 300-ppm exposure

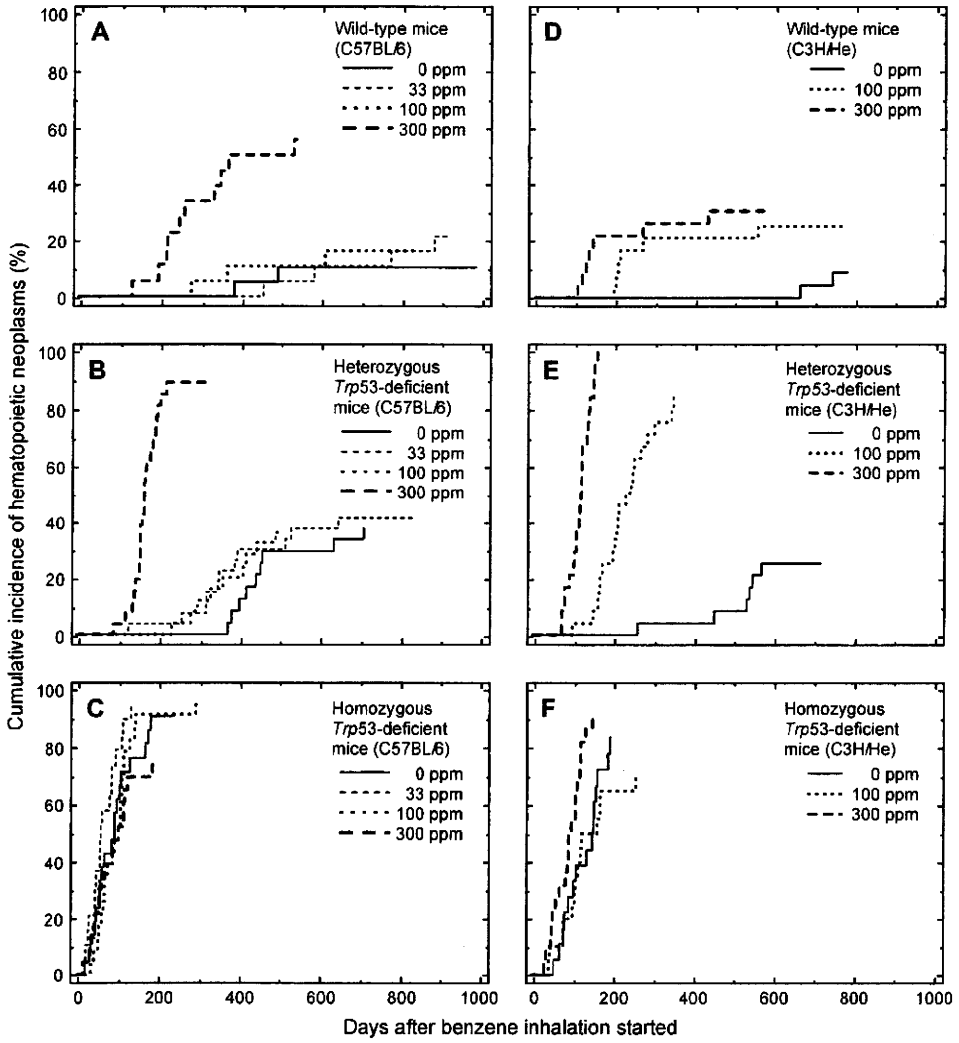

FIG. 2. The illustration shows the cumulative lifetime incidence of all hematopoietic malignancies (%) in C57BL/6 mice on the left (A–C) and in C3H/He mice on the right (D–F), using (A) and (D) for wild-type mice, (B) and (E) for heterozygous p53-deficient mice, and (C) and (F) for homozygous p53-deficient mice. Bold dotted line for 300-ppm exposure group, regular dotted line for 100-ppm exposure group, fine dotted line for 33-ppm exposure group, and solid line for sham exposure control. Statistical significance by log-rank test: (A) 0 versus 300 ppm, $p = 2.7 \times 10^{-04}$; 33 versus 300 ppm, $p = 4.5 \times 10^{-04}$; 100 versus 300 ppm, $p = 1.8 \times 10^{-03}$; (B) 0 versus 300 ppm, $p = 2.5 \times 10^{-10}$; 33 versus 300 ppm, $p = 8.6 \times 10^{-10}$; 100 versus 300 ppm, $p = 9.1 \times 10^{-10}$; (C) no significant difference between groups; (D) 0 versus 300 ppm, $p = 7.0 \times 10^{-03}$; (E) 0 versus 300 ppm, $p = 1.2 \times 10^{-11}$; 100 versus 300 ppm, $p = 2.8 \times 10^{-09}$; 0 versus 100 ppm, $p = 4.2 \times 10^{-08}$; and (F) 0 versus 300 ppm, $p = 3.1 \times 10^{-03}$; 100 versus 300 ppm, $p = 2.1 \times 10^{-02}$.

group showed a significant differences in cumulative HPN incidence in comparison to the other C57BL/6 groups. However, findings from both the C3H/He 300-ppm and the 100-ppm exposure groups differed significantly from the sham exposure controls. These results imply that HPNs occurred at a higher than threshold level in heterozygous *Trp*53-deficient mice in both strains since such incidence was greater than and clearly separated from the incidence in each sham exposure control groups.

Exposure in Trp53-deficient mice. A high frequency of HPNs was observed in both strains of the heterozygous *Trp*53-deficient benzene exposure groups as shown in Figure 2B (C57BL/6) and Figure 2E (C3H/He). In heterozygous *Trp*53-deficient C57BL/6 mice, a total HPN incidence of 88.5% (300 ppm) was observed from 88 to 219 days. This incidence was higher than in the sham exposure control (37.5%) and also higher than in the wild-type groups with or without benzene exposure (55.6 and 10.0%, respectively) and with earlier onset time (88 days) than in wild-type mice (130 days). The increase in incidence of HPNs between benzene exposure group and sham control was not greater in *Trp*53-deficient C57BL/6 mice than in the wild-type mice due to an increase in the late-appearing spontaneous HPNs in the *Trp*53-deficient mice, but the 50% die-off time (days) for HPNs between the former and

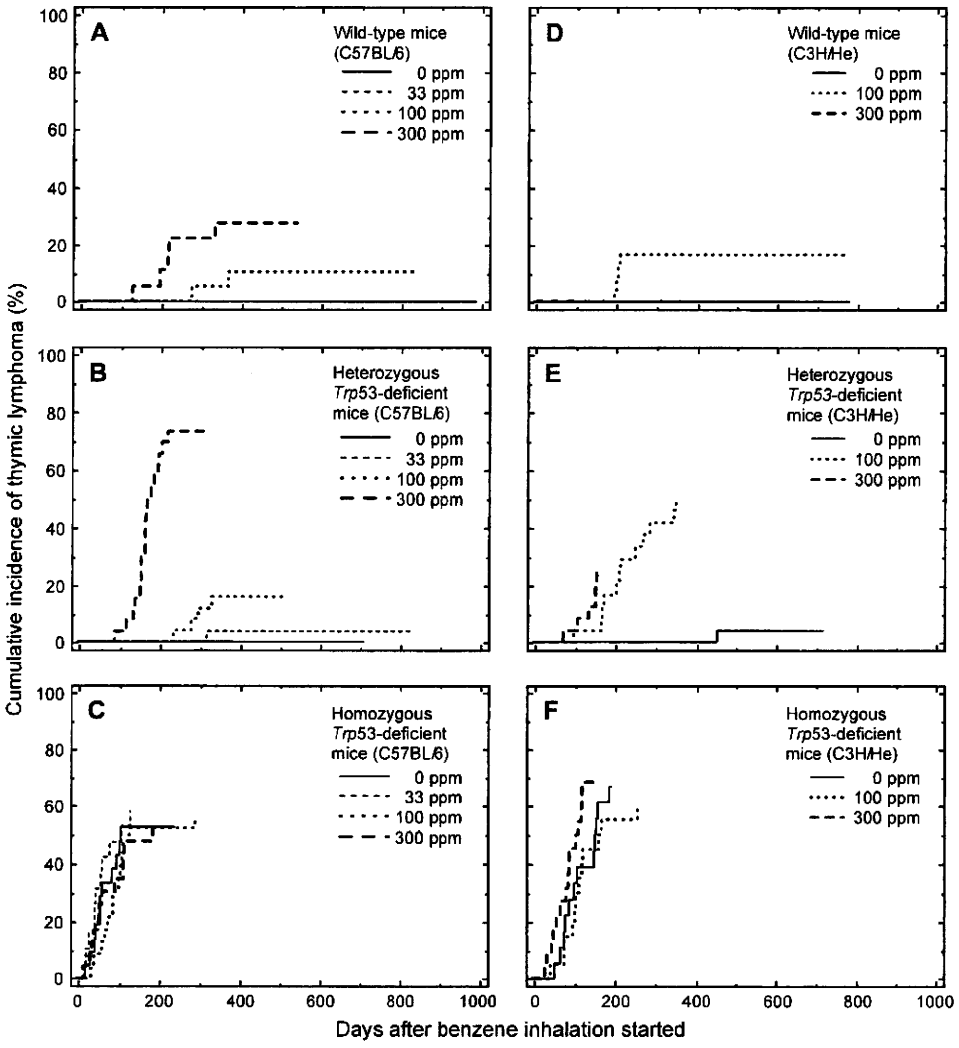

FIG. 3. Cumulative lifetime incidences of thymic lymphoma during the lifetime in C57BL/6 mice on the left (A–C) and C3H/He mice on the right (D–F), using (A) and (D) for the wild-type mice, (B) and (E) for heterozygous *Trp*53-deficient mice, and (C) and (F) for homozygous *Trp*53-deficient mice. Bold dotted line for 300-ppm exposure group, regular dotted line for 100-ppm exposure group, fine dotted line for 33-ppm exposure group, and solid line for sham exposure control. Statistical significance by log-rank test: (A) 0 versus 300 ppm, $p = 9.7 \times 10^{-03}$; 33 versus 300 ppm, $p = 1.2 \times 10^{-02}$; (B) 0 versus 300 ppm, $p = 4.3 \times 10^{-10}$; 33 versus 300 ppm, $p = 1.7 \times 10^{-10}$; 100 versus 300 ppm, $p = 6.6 \times 10^{-09}$; 0 versus 100 ppm, $p = 3.4 \times 10^{-02}$; (C) no significant difference between the two groups; (D) 0 versus 100 ppm, $p = 4.8 \times 10^{-02}$; (E) 0 versus 300 ppm, $p = 3.9 \times 10^{-05}$; 100 versus 300 ppm, $p = 2.4 \times 10^{-03}$; 0 versus 100 ppm, $p = 2.4 \times 10^{-06}$; and (F) 0 versus 300 ppm, $p = 3.0 \times 10^{-02}$.

the latter was significantly split in the *Trp*53-deficient mice than in the wild-type mice (266.5 vs. 184.5 days). The cumulative incidence curves for HPN in these heterozygous *Trp*53-deficient exposure groups were significantly different only in the 300-ppm exposure group, and the curves of the remaining groups occasionally overlapped for the C57BL/6 strain, but the benzene dose–dependent shortening of 50% die-out time in the 100-ppm group and the die-out time in the 100-ppm *Trp*53-deficient groups were both similarly reduced (70.5 and 70.0 days).

In heterozygous *Trp*53-deficient C3H/He mice, in contrast, the total incidence of HPNs increased in a manner dependent on the benzene exposure dose (104.2, 83.3, and 25.0%, respectively), with earlier onset times (78, 98, and 260 days) than in wild-type mice (105, 197, and 651 days).

As illustrated in Figure 2C (C57BL/6) and Figure 2F (C3H/He), although homozygous *Trp*53-deficient mice showed slightly earlier onset of thymic lymphomas following benzene exposure, specifically in the C3H/He strain, these mice were not used for bioassay because they showed extremely early onset of highly frequent thymic lymphomas that developed spontaneously by a known mechanism, that is, development of CD4/CD8 double-negative thymic lymphomas owing to the

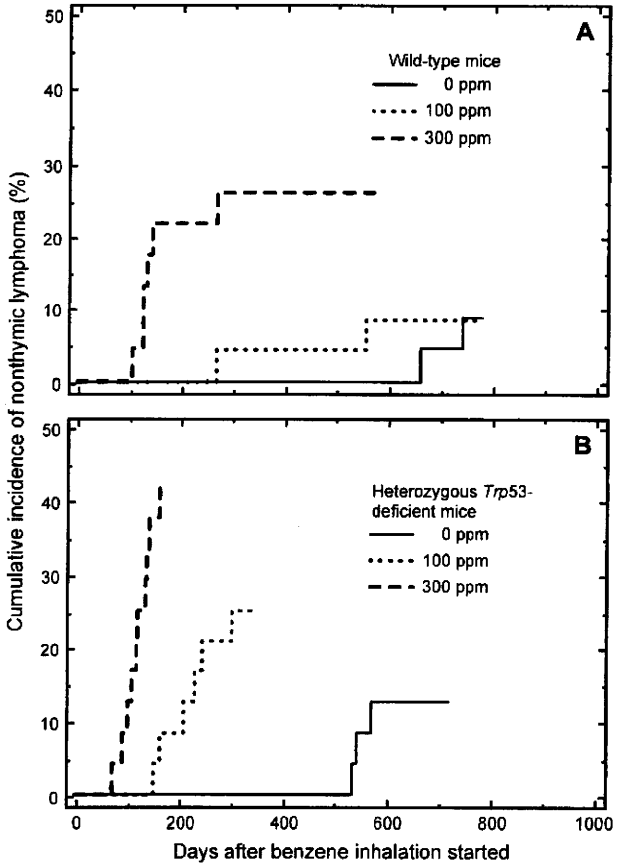

FIG. 4. Cumulative lifetime incidence of nonthymic (non-Hodgkin) lymphoma in C3H/He mice; wild-type mice (A) and heterozygous *Trp*53-deficient mice (B). Bold dotted line for 300 ppm, regular dotted line for 100 ppm, and solid line for sham exposure control. Statistical significance determined by log-rank test: (A) 0 versus 300 ppm, $p = 3.1 \times 10^{-02}$; and (B) 0 versus 300 ppm, $p = 1.4 \times 10^{-05}$; 100 versus 300 ppm, $p = 6.4 \times 10^{-05}$; 0 versus 100 ppm, $p = 4.0 \times 10^{-03}$.

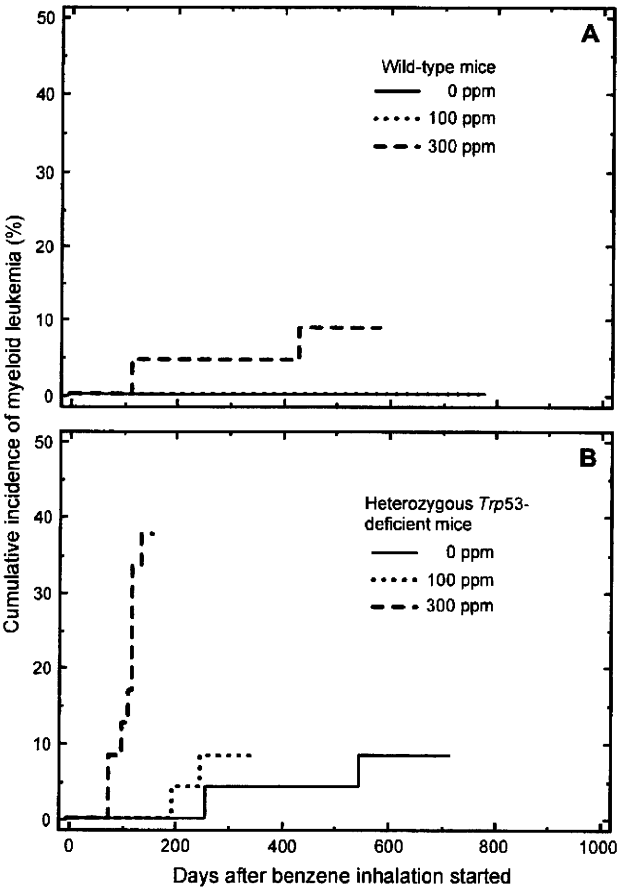

FIG. 5. Cumulative lifetime incidences of myelogenous leukemia during the lifetime of C3H/He mice; wild-type mice (A) and heterozygous *Trp*53-deficient mice (B). Bold dotted lines for 300 ppm, regular dotted lines for 100 ppm, and solid lines for sham exposure controls. Statistical significance determined by log-rank test: (A) no significant difference between groups; (B) 0 versus 300 ppm, $p = 1.5 \times 10^{-04}$; 100 versus 300 ppm, $p = 1.8 \times 10^{-04}$.

absence of physiological apoptosis in the CD4/CD8 double-negative immature T-cell population during early development (Haines *et al.*, 2006).

Histopathological Examination

HPNs, along with non-HPNs and non-neoplastic diseases observed in C57BL/6 mice and C3H/He mice, were classified histopathologically and tabulated separately in Table 1 for the C57BL/6 strain and Table 2 for the C3H/He strain.

Development of thymic and nonthymic lymphoma. As shown in these tables, in wild-type mice, only a small number of HPNs, that is, thymic lymphomas, two (10.5%) and five (27.8%) in the C57BL/6 and four (16.7%) and zero (0%) in the C3H/He, were observed in 100- and 300-ppm exposure groups, respectively (Figs. 3A and 3D). In heterozygous *Trp*53-deficient C57BL/6 mice, the number of thymic lymphomas gradually increased, that is, 0, 1 (3.7%), 4 (16.0%), and 19 (73.1%), with benzene exposure dose, that is, 0, 33, 100, and 300 ppm, respectively (Fig. 3B). Thus, the graded increase in the incidence of thymic lymphomas up to 73.1% was observed in the C57BL/6 strain, showing a linear exposure dose-response relationship. In C3H/He mice, on the other hand, the number of thymic lymphomas that developed were 1 (4.2%), 12 (50.0%), and 6 (25.0%) at benzene exposure doses of 0, 100, and 300 ppm, respectively (Fig. 3E). Thus, the number of thymic lymphomas at 300 ppm decreased and a linear exposure dose-response relationship was not observed. The mechanism underlying this observation needs to be studied.

Concerning the incidence of nonthymic (non-Hodgkin) lymphomas, a linear exposure dose-response relationship was not observed in the C57BL/6 strain, but relative increases in the incidence with the exposure dose of benzene were observed in C3H/He mice (Figs. 4A and 4B).

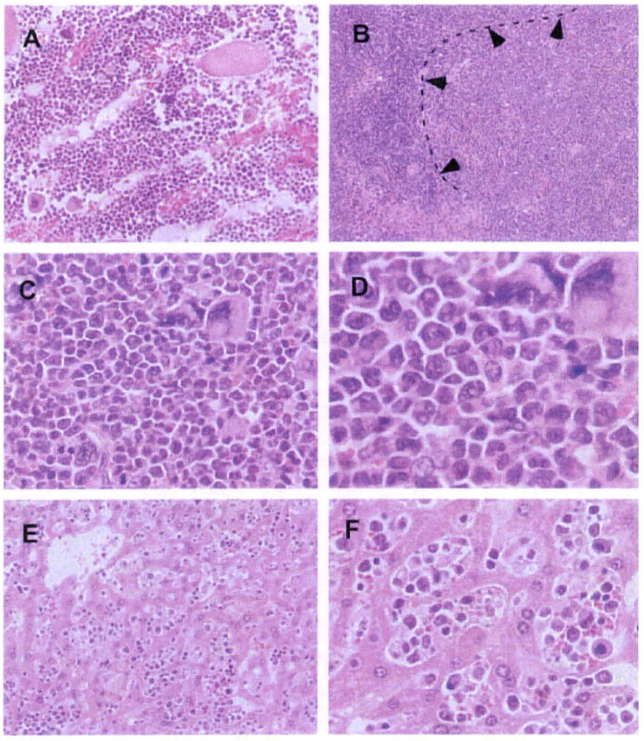

FIG. 6. Representative histopathological findings for AMLs developing in benzene-exposed wild-type C3H/He mice (A–F). Increased cellular density for atypical mononuclear cells with heterogeneous size distribution in sternum BM (A, ×67). Increased cellular density with expanding growth (arrow heads) toward surrounding splenic tissue and the lymphofollicular structures on the left (B, ×34). Higher magnification of neoplastic cellular component at the center of leukemic growth in (B), consisting of atypical myeloid cells with scattered bizarrely shaped myeloblastic nuclei including megakaryocytes (C, ×169). Higher magnification of (C), revealing detailed atypical myeloid cells, including cells with occasional doughnut-shaped nuclei (D, ×312). Hepatic cell cord filled with atypical mononuclear myeloblastic cell component surrounding a central vein at the upper left (E, ×67). Higher magnification of (E), including atypical myeloid cells, with heterogeneous size distribution, proliferating in sinusoidal spaces (F, ×169).

Development of AMLs. It is notable that heterozygous *Trp*53-deficient C3H/He mice, which are prone to AML, produced two (8.3%), two (8.3%), and nine (37.5%) AMLs in the 0-, 100-, and 300-ppm exposure groups, respectively, in comparison with wild-type mice, which produced only two (8.7%) AMLs in the 300-ppm exposure group (Fig. 5). In C57BL/6 mice, there were two AMLs in heterozygous and one in homozygous *Trp*53-deficient animals. There were essentially no significant differences in cytological and histopathological findings of AMLs between the both strains. Thus, mainly cytological and histopathological findings of AMLs developed in C3H/He mice are shown in Figure 6 (leukemias developing in wild-type mice) and Figure 7 (leukemias developing in *Trp*53-deficient mice), along with two panels (7E and 7F) from heterozygous *Trp*53-deficient C57BL/6 mice in Figure 7, bottom.

In Figure 6, atypical myeloblastic leukemic cells with irregularly bizarrely shaped nuclei, occasionally including doughnut-shaped nuclei as shown in Figures 6C and 6D, suggest a myelogenous origin in C3H/He mice. The same atypical myeloid cells with a heterogeneous size distribution were observed to invade hepatic sinusoidal spaces (Figs. 6E and 6F). In wild-type mice, AMLs developed only in the C3H/He mice and not in the C57BL/6 mice.

Owing to the function of *Trp*53 during the early developmental stage, a prominently lesser extent of differentiation was noted in AMLs developing in *Trp*53-deficient mice. Namely, as shown in Figure 7, the cytopathological and histopathological characteristics of leukemic cells in both heterozygous *Trp*53-deficient C3H/He mice (Figs. 7A–D) and C57BL/6 mice (Fig. 7E) revealed more immature blastic cells with less differentiation than leukemic cells in wild-type mice (Fig. 6). Representative atypical myeloblastic cells possessing trace peroxidase granules in the cytoplasm are shown in Figure 7B (inset, bottom). Nevertheless, some doughnut-shaped nuclei similar to those of cells with myeloid lineages were very occasionally observed in the C57BL/6 strain (Fig. 7E, inset, top and bottom).

HPNs in Relationship to Benzene Exposure Dose

The exposure dose range for benzene hematotoxicity is narrow, specifically for the induction of HPNs. Higher benzene exposure may produce a larger number of hematopoietic neoplastic candidates but simultaneously seems to decrease the number of hematopoietic progenitor cells, that is, potential targets for the induction of HPNs. Figures 8A and 8B (for C57BL/6 mice) and Figures 8C and 8D (for C3H/He mice) illustrate the relationship between the incidence of HPNs and graded increased benzene exposure.

In C57BL/6 mice, the increase in the total incidence of HPNs was only significant in both the 300-ppm exposure groups for wild-type and the heterozygous *Trp*53-deficient mice. Each histological type showed a statistically significant increase in the incidence of thymic lymphoma at 300-ppm exposure in comparison to sham exposure (Table 1). There was no statistically significant increase in HPN incidence in either the 33- or 100-ppm exposure group in comparison to spontaneous HPNs in the sham exposure groups, possibly due to the competitive increase in the incidence of non-HPNs.

In the C3H/He mice, however, the total incidence curve for HPNs in wild-type mice showed a gradual increase reaching a plateau/peak in the wild-type 100- and 300-ppm exposure groups (Fig. 8C). The heterozygous *Trp*53-deficient mice showed a significant increase (*) in HPN incidence in the 100- and 300-ppm exposure groups, reaching up to 100% in the latter group (Fig. 8D).

DISCUSSION

In this research, we sought answers to three questions. For the first question regarding the equivocal induction of HPNs at

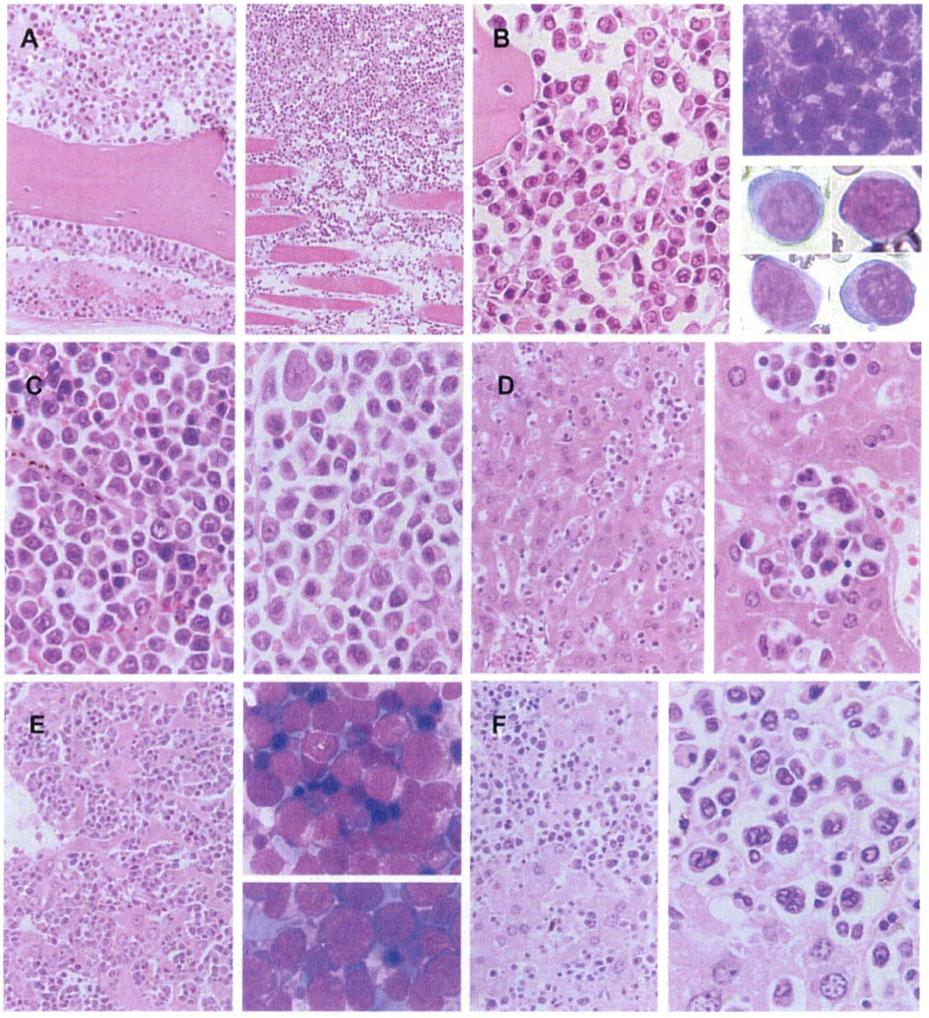

FIG. 7. Representative histopathological and cytopathological findings for AML developing after benzene exposure in heterozygous *Trp*53-deficient C3H/He and C57BL/6 mice: AML in femoral BM (A, left ×67) and its periosteal, intramuscular expansion, and infiltration into growth against surrounding soft part of femoral bone (A, right ×34). Higher magnification of atypical myeloid cells with widely heterogeneous size distribution and marked cellular atypia (B, left ×169). Imprint smear of hyperchromatic myeloblastic cells (B, inset top ×253) and representative characteristics of leukemic cells in smear showing atypical immature myeloblastic cells with trace evidence of intracytoplasmic peroxidase granulation (B, inset, upper row ×494 and lower row ×643). Atypical myeloid cellular component, proliferating in splenic white pulp for *Trp*53-deficient C3H/He mice (C, left ×169) and at higher magnification (C, right ×253). Hepatic trabecular infiltration of myeloid cells in liver of *Trp*53-deficient C3H/He mice (D, left ×67) and at higher magnification (D, right ×169). Atypical myeloid cell proliferation in liver of *Trp*53-deficient C57BL/6 mice (E, left ×67), atypical immature myeloid cells (E, right, top ×337), and tissue imprint smear from terminal stage of spleen with immature mononuclear myeloblastic cells (E, right, bottom ×337). Representative nonthymic malignant lymphoma, infiltrating into hepatic sinusoidal spaces (F, left ×67) with higher magnification of expansive growth of cerebriform bizarrely shaped cells (F, right ×169) in *Trp*53-deficient C57BL/6 mice.

low dose of benzene exposure, we found that heterozygous *Trp*53-deficient mice in both strains showed a higher than threshold incidence of HPNs at lower doses, as described in the "Results" section. We attribute this to the mechanism of *Trp*53-dependent repair for DNA damage induced by benzene exposure. Our second question related to the nonlinear plateau in the incidence of HPNs at high dose of benzene exposure. We found that *Trp*53-deficient mice in both strains produced a fairly high incidence of HPNs up to 100%, including 38% of AMLs in C3H/He mice exposed to benzene 300 ppm in comparison with an incidence of only 9% in wild-type mice exposed to the same dose. These results suggest that the nonlinear plateau in the incidence of HPNs at high benzene exposure may be caused by a decrease in neoplastic target cells due to *Trp*53-dependent escape from apoptosis in wild-type mice. In addition to benzene-mediated genotoxicity, the development of HPNs generally requires an epigenetic process that does not exhaust but maintains hematopoietic stem/progenitor cells, that is, the target cells for hematopoietic neoplastic development. An excessive decrease in the number

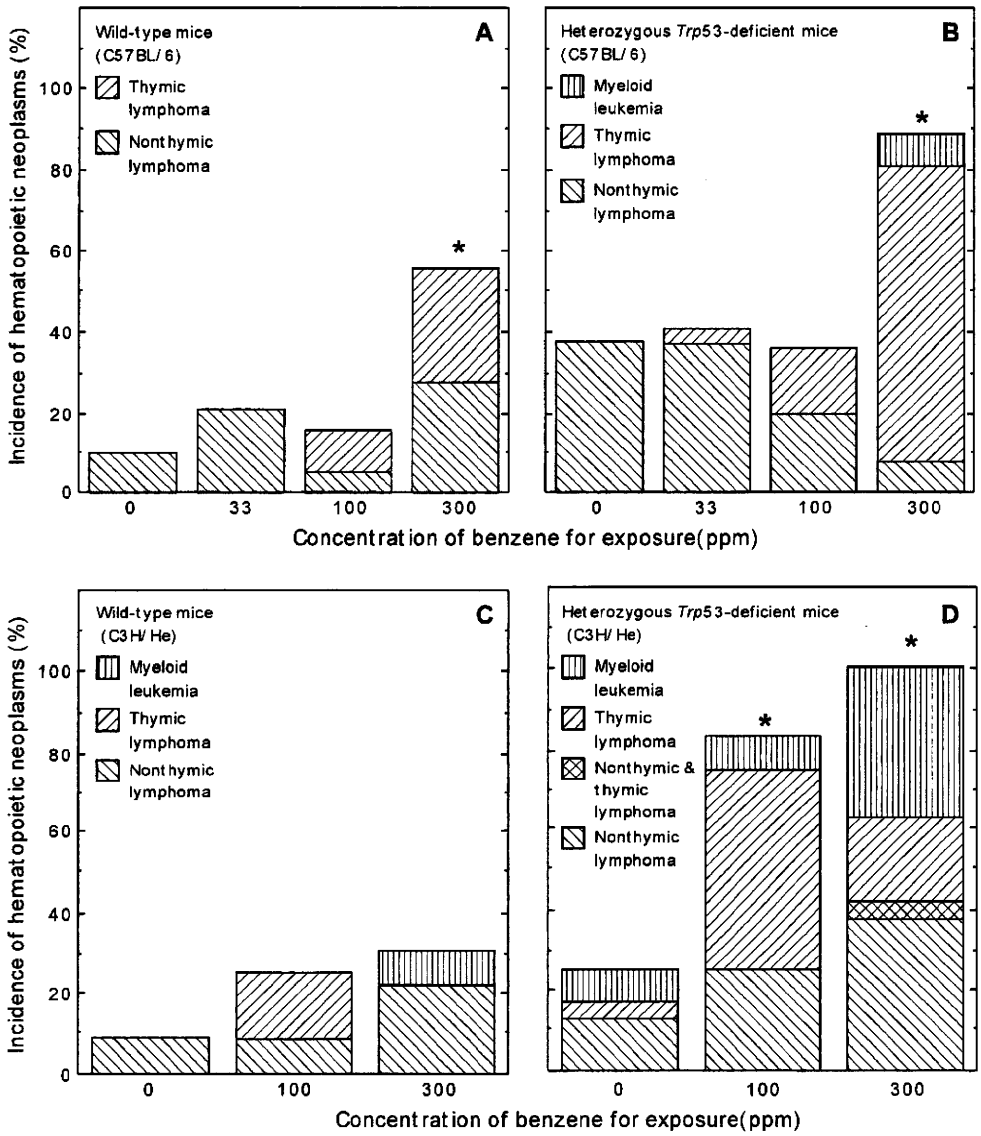

FIG. 8. Incidences of HPN histological types are shown in bar graphs. (A, B) C57BL/6 strain, wild-type mice (A) and heterozygous *Trp*53-deficient mice (B); (C, D) C3H/He strain, wild-type mice (C) and heterozygous *Trp*53-deficient mice (D). The incidence of HPNs is shown on the ordinate axis versus benzene exposure dose for the C57BL/6 strain (0, 33, 100, and 300 ppm) or the C3H/He strain (0, 100, and 300 ppm) on the horizontal axis of each graph. Histological types, such as AML, thymic lymphoma, nonthymic lymphoma associated with thymic lymphoma, and nonthymic lymphoma, are designated by inset legends in each figure. Incidences in heterozygous *Trp*53-deficient mice are higher than those in wild-type mice. Incidences in the 300-ppm exposure only (*) show statistically significant differences for both wild-type mice and heterozygous *Trp*53-deficient mice.

of hematopoietic stem/progenitor cells does not induce any hematopoietic neoplastic growth but rather induces irreversible aplastic anemia (Cronkite *et al.*, 1982). The Snyder-Cronkite benzene exposure protocol of 300 ppm, 6 h/day, 5 days/week, for the animal's lifetime or 16 weeks was originally aimed to not exhaust but maintain hematopoietic stem/progenitor cells. The exposure period was subsequently extended for the protocols up to 2 years in length (Huff *et al.*, 1989; NTP, 1986), but no substantial increase in the incidence of observed HPNs was reported. The exposure period applied in the present study was longer than in the original protocol by Cronkite *et al.* (1984, 1985, 1989) (16 weeks), which produced a higher incidence of HPNs owing to less exhaustion of hematopoietic stem/progenitor cells even in wild-type mice in both C57BL/6 and C3H/He strains. The relationship between the incidence of HPNs and the benzene exposure dose, however, showed a maximum increase to plateau with benzene exposure at less than 300 ppm (Figs. 2A and 2D). It, thus, appears that the

number of stem/progenitor cells available for targeting at 300 ppm in C3H/He mice is practically marginal not only for thymic lymphomas but also for all HPNs.

The potential for inducing HPNs seems to be limited in wild-type mice, as shown by the present protocol in both C57BL/6 and C3H/He strains as well as in reports by Huff *et al.* (1989) and the NTP (1986). However, we noted enhanced induction of HPNs after benzene exposure in *Trp*53-deficient mice and attributed this to arrest of the stem cell–specific cell cycle possibly owing to the genotoxicity induced by benzene exposure. Moreover, owing to *Trp*53 deficiency, benzene exposure in excess of 300 ppm appears to suppress the induction of HPNs as evidenced by the incidence of thymic lymphomas in heterozygous *Trp*53-deficient mice (Fig. 8D). A nonlinear limited increase and plateaued increase in the incidence of HPNs were also confirmed for the higher incidence of HPNs in *Trp*53-deficient mice with an impaired repair system. Regarding the known association between lower benzene toxicity and higher LD_{50} values, the results imply a trend based on the possible loss of progenitor cell–specific target cells for HPNs, that is, hematopoietic progenitor cells at higher benzene exposures (Yoon *et al.*, 2002).

*Trp*53-deficient mice develop undifferentiated immature HPNs (Yoshida *et al.*, 2002), which are attributed to the failure of *Trp*53 expression to regulate the differentiation process in myeloid cells (Feinstein *et al.*, 1992; Kastan *et al.*, 1991; Skorski *et al.*, 1996; Soddu *et al.*, 1994). As reported previously for radiation-induced AML in *Trp*53-deficient mice (Yoshida *et al.*, 2002, 2007), such AML tends to be characterized by a high incidence of stem cell leukemias and/or blastic leukemias, and there are traces of myeloid differentiation in homozygous *Trp*53-deficient mice with or without radiation exposure. Interestingly, the leukemia developing in *Trp*53-deficient mice after benzene exposure also showed less differentiation in the present study. Such reductions in differentiation are not seen in other thymic or nonthymic lymphomas. However, we were unable to confirm those findings here owing to insufficient data analysis of the precise level of differentiation since differentiation biomarkers for thymic and nonthymic lymphomas were not applied in the present study.

Third, the last issue is why benzene-induced HPNs are not leukemic, but largely thymic and nonthymic lymphomatous in mice (Cronkite *et al.*, 1985; Huff *et al.*, 1989), whereas most of the HPNs that develop after benzene exposure in humans are AMLs (Aksoy *et al.*, 1974; Delore and Borgomano, 1928; Vigliani and Forni, 1976). This query relating to the experimental development of leukemias in the narrow exposure dose range of benzene-induced HPNs has not been satisfactorily answered to date. In the present study, we found a marked difference between C57BL/6 and C3H/He mice in the incidence of different types of HPNs. Specifically, thymic lymphomas were predominantly induced in C57BL/6 mice, whereas nonthymic lymphomas were predominantly induced in C3H/He mice. Our findings may be supported by the gene expression differences reported for these strains after benzene exposure since the gene expression profiles in both strains were, to some extent, reciprocal for some cell cycle–regulating genes (data not shown). Comparable differences were also observed in the incidence of AMLs. Similar to findings following radiation exposure, C3H/He mice, which are prone to developing AMLs, tended to develop AMLs following benzene exposure.

An exposure-dependent limited increase was again observed in the incidence of AMLs up to 37.5% in *Trp*53-deficient C3H/He mice, and AMLs also developed even in wild-type C3H/He mice when exposed to 300 ppm. However, only two *Trp*53-deficient C57BL/6 mice developed AML at 300 ppm. This implies that there is a potential leukemogenicity not only in the C3H/He strain but also in the C57BL/6 strain, although in the C3H/He strain such leukemogenicity is associated more with an as-yet–undefined genetic background for induction of AMLs.

We noted a few C57BL/6 mice with myeloproliferative and/or myelodysplastic syndrome in the 33-ppm exposure group. This suggests that the protocol of 33-ppm exposure was insufficient for inducing HPNs since these syndromes are considered to be a preleukemic hematopoietic disorder.

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Quality assessment of an interferon-gamma release assay for tuberculosis infection in a resource-limited setting

Nguyen TL Hang[1], Naoki Ishizuka[2], Naoto Keicho*[2], Le T Hong[3], Do B Tam[3], Vu TX Thu[3], Ikumi Matsushita[2], Nobuyuki Harada[4], Kazue Higuchi[4], Shinsaku Sakurada[2] and Luu T Lien[3]

Address: [1]IMCJ-BMH Medical Collaboration Center, Bach Mai Hospital, Hanoi, Viet Nam, [2]International Medical Center of Japan, Research Institute, Tokyo, Japan, [3]Hanoi Tuberculosis and Lung Disease Hospital, Hanoi, Viet Nam and [4]Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo, Japan

Email: Nguyen TL Hang - lehang0310@gmail.com; Naoki Ishizuka - naishi@ri.imcj.go.jp; Naoto Keicho* - nkeicho-tky@umin.ac.jp; Le T Hong - lethihong.hth@gmail.com; Do B Tam - dobangtam.hth@gmail.com; Vu TX Thu - vuthixuanthu.hth@gmail.com; Ikumi Matsushita - ikumi-tky@umin.ac.jp; Nobuyuki Harada - harada@jata.or.jp; Kazue Higuchi - higuchi@jata.or.jp; Shinsaku Sakurada - ssakura@mti.biglobe.ne.jp; Luu T Lien - luuthilien.hth@gmail.com

* Corresponding author

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Abstract

Background: When a test for diagnosis of infectious diseases is introduced in a resource-limited setting, monitoring quality is a major concern. An optimized design of experiment and statistical models are required for this assessment.

Methods: Interferon-gamma release assay to detect tuberculosis (TB) infection from whole blood was tested in Hanoi, Viet Nam. Balanced incomplete block design (BIBD) was planned and fixed-effect models with heterogeneous error variance were used for analysis. In the first trial, the whole blood from 12 donors was incubated with nil, TB-specific antigens or mitogen. In 72 measurements, two laboratory members exchanged their roles in harvesting plasma and testing for interferon-gamma release using enzyme linked immunosorbent assay (ELISA) technique. After intervention including checkup of all steps and standard operation procedures, the second trial was implemented in a similar manner.

Results: The lack of precision in the first trial was clearly demonstrated. Large within-individual error was significantly affected by both harvester and ELISA operator, indicating that both of the steps had problems. After the intervention, overall within-individual error was significantly reduced ($P < 0.0001$) and error variance was no longer affected by laboratory personnel in charge, indicating that a marked improvement could be objectively observed.

Conclusion: BIBD and analysis of fixed-effect models with heterogeneous variance are suitable and useful for objective and individualized assessment of proficiency in a multistep diagnostic test for infectious diseases in a resource-constrained laboratory. The action plan based on our findings would be worth considering when monitoring for internal quality control is difficult on site.

Background

Assuring quality is essential for clinical laboratories in the field of infectious diseases. Beneficiaries are not only patients obtaining a diagnosis on site but also future patients receiving benefits of clinical research supported by qualified laboratories. Quality assurance in modern laboratories is realized by total quality management including external quality assurance (EQA) and internal quality control (IQC) [1-3].

In most resource-constrained countries, however, regulations on quality assurance have not been laid down by the authorities and accuracy and precision of clinical measurements have not been monitored systematically [4]. Under such disadvantageous circumstances, when important but rather complicated testing for infectious diseases is undertaken, we cannot easily be confident that the skill has been transferred and maintained properly until the procedure becomes familiar and stably performed in accordance with a desirable quality control system [5]. During this vulnerable period, how to assess proficiency of the testing effectively and objectively, and how to assure and improve the quality are open issues to be addressed.

Currently, immunoassay is commonly used to make a serological diagnosis of infectious diseases involving human immunodeficiency virus, a variety of hepatitis virus and other sexually transmitted or blood-borne pathogens [6,7], which are serious problems in the developing world. Enzyme linked immunosorbent assay (ELISA) is often used to make diagnosis of these diseases in the clinical laboratories. Because of the complexity of the method, however, quality control of these assay systems is challenging [8]. In this context, trend of point of care (POC) tests that facilitate immediate and on-site diagnosis as well as early treatment of infectious diseases has been emphasized [7]. However, their usage in resource-constrained countries is still hampered by high cost and difficulties in testing for high throughput screening and thus laboratory-based immunoassays would be irreplaceable in many fields.

Recently, a two-step immunoassay to detect tuberculosis (TB) infection has also been developed and used extensively [9]. It consists of whole blood stimulation with TB-specific antigens followed by measurement of interferon-gamma using ELISA. Our objective in the present study is to demonstrate that the quality of laboratory tests can be assessed objectively even in a resource-constrained laboratory if the optimum design of experiments and appropriate statistical models are chosen. As a result of this attempt, we experienced marked improvement of the quality of this multi-step immunoassay made by more than one laboratory staff member in a hospital of Viet Nam. We proposed a general plan to evaluate skills of laboratory staff members efficiently and quantitatively to perform qualified immuno-diagnostic testing especially for infectious diseases until such time as they establish a total quality management system by themselves.

Methods

Interferon-gamma release assay (IGRA) for diagnosis of TB infection

IGRA is a general method to measure interferon-gamma induced by *Mycobacterium tuberculosis*-specific antigens (TB-Antigen) for detecting TB infection. In the ELISA-based IGRA (QuantiFERON-TB Gold In-Tube™, Cellestis, Victoria, Australia), one milliliter of the whole blood was collected into the Nil tube for negative control, Mitogen for positive control, and TB-Antigen separately. The blood in the tubes was mixed and placed in the incubator for 18 hours at 37°C (Cool incubator NC-25B, Funakoshi, Tokyo, Japan). Approximately 200 μl of plasma were harvested from each tube after centrifugation (Kubota 2010, Kubota, Tokyo, Japan).

Interferon-gamma concentrations in the plasma were measured by ELISA, using microtiter plate washer and reader (Wellwash Plus Microplate Washer and Multiscan JX Microplate Reader, Thermo Electron Corporation, Vantaa, Finland) with the analysis software provided by the manufacturer (QuantiFERON-TB Gold Analysis Software, ver. 2.50, Cellestis). In this study, interferon-gamma concentrations obtained from this calculation were directly used for further analysis.

Study setting

Two trials were carried out in Hanoi TB and Lung Disease Hospital, Viet Nam. Between the first and second trial, statistical analysis was made and an intervention was planned to ensure counterchecking and correct questionable manipulations. Each trial consisted of two runs. In each run, three milliliters of blood were collected from volunteers after informed consent had been obtained. Study protocols using IGRA were approved by ethical committees of the Ministry of Health, Viet Nam and International Medical Center of Japan respectively.

Two laboratory members, A and B, performed either plasma harvest or ELISA operation or both: Harvest included labeling and placing plasma storage tubes properly and transferring plasma from centrifuged blood collection tubes to these tubes by pipetting. ELISA was a process including preparing reagents and transferring plasma samples into the microtiter plate. ELISA ended with calculation of interferon-gamma concentration. Because their roles were changed occasionally due to the limited manpower of the laboratory, their performance in both Harvest and ELISA was the subject to be analyzed.

Balanced incomplete block design (BIBD)

A single specimen obtained from routine blood collection was not sufficient to assess two staff members' performance. Because additional blood sampling was not easily accepted in many countries including Viet Nam, BIBD was attempted to obtain analytical information from small volume of plasma samples in this study: Of four possible combinations of harvester and ELISA operator, two combinations were cyclically chosen, using the limited amount of specimen. Allocation of observed combinations by BIBD in this study was described in Table 1. In each trial, there were two levels of Harvest (two different Harvesters), two levels of ELISA (two different ELISA operators) and 12 levels of Specimen (12 different blood donors).

Outliers

To identify outliers, Mahalanobis distance D was calculated, which took the distance from the mean and correlation into account [10]. When D > 2.0, the value of that observed pair was regarded as outlier.

A fixed-effect model and three-way analysis of variance (ANOVA)

To assess effects of factors of interest and error variance, we used a fixed-effect model:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijk}$$

of which,

y_{ijk}: Interferon-gamma concentration in the plasma

μ: Grand mean of all measurements

α_i: Harvest with i levels: i = 1, 2 (= A and B)

β_j: ELISA with j levels: j = 1, 2 (= A and B)

γ_k: Blood specimen with k levels: k = 1, 2,..., 12

ε_{ijk}: Within-individual error; following normal distribution with mean = 0 and variance = σ^2: N (0, σ^2)

In this clinical setting, effects of interaction terms were not considered in the above model, because harvesting plasma and performing ELISA are independent steps and it is unlikely that the exchanging of staff roles in itself could increase the chances of error.

Analysis of heterogeneous error variance affected by a given factor

To determine whether individuals of Harvest or ELISA affect within-individual error, we assessed a fixed-effect model with heterogeneous variance of error in the following way:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ij}$$

where error follows the normal distribution $N(0, \sigma_{ij}^2)$.

Error variance affected by Harvesters was evaluated in the following formula:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{i.}$$

Similarly, the following formula was used for error variance affected by ELISA operators:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{.j}$$

Coefficient of variation (CV) before and after intervention

Error variance ε_{ijk} that included sources of Harvest and ELISA was calculated in a simple one-way ANOVA model adjusted by specimen. Based on the following formula, CVs of the two trials were assessed:

$$y = \mu + \gamma_k + \varepsilon_{ijk}$$

Table 1: Allocation of observed combinations of Harvester and ELISA operator.

Sample	Specimen*	Harvest	ELISA	Data	Sample	Specimen	Harvest	ELISA	Data
1	1	A	A	Observed	7	4	A	A	Observed
2	1	A	B	Observed		4	A	B	Not observed
	1	B	A	Not observed	8	4	B	A	Observed
	1	B	B	Not observed		4	B	B	Not observed
	2	A	A	Not observed		5	A	A	Not observed
	2	A	B	Not observed	9	5	A	B	Observed
3	2	B	A	Observed		5	B	A	Not observed
4	2	B	B	Observed	10	5	B	B	Observed
5	3	A	A	Observed		6	A	A	Not observed
	3	A	B	Not observed	11	6	A	B	Observed
	3	B	A	Not observed	12	6	B	A	Observed
6	3	B	B	Observed		6	B	B	Not observed

*To each specimen, two measurements were assigned. This layout was repeated twice by using different sets of specimens in each trial.

$$CV(\%) = \frac{\text{Root mean square error}}{\text{Mean}} \times 100$$

CV should not be larger than 20% in any types of immunoassay [8].

Assessment of heterogeneous variance between the two trials

To analyze overall within-individual error between the two trials statistically, we used a fixed-effect model with heterogeneous variance of error, under the assumption that α and β were fixed throughout the trials. The effect of each blood specimen γ was expected to be different between the two trials.

$$y_{ijk1} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijk1} \text{(the first trial)}$$
$$\| \quad \| \quad \not\| \quad \not\|$$
$$y_{ijk2} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijk2} \text{(the second trial)}$$

of which, ε_{ijk1} and ε_{ijk2} were within-individual errors of the first and the second trial respectively. On the above assumption, ε_{ijk1} and ε_{ijk2} would be heterogeneous error between the trials.

Calculation of Mahalanobis distance, three-way ANOVA and estimation of heterogeneous variance were performed by SAS version 9.1 (SAS Institute Cary, NC, USA). Differences in error variance of two trials and error variance affected by a given factor were considered to be significant when *P*-value was less than 0.05.

Results

Evaluation of outliers and three-way ANOVA in the first trial

Out of 72 measurements obtained from the first trial, seven outliers were identified: One was in Nil condition, three in TB-Antigen and three in Mitogen (Mahalanobis D = 2.64 to 4.69).

To assess effects of individuals for Harvest and ELISA and character of errors involved in the first trial, we first performed three-way ANOVA using a fixed-effect model, in which three factors, Harvest, ELISA and individual blood specimens may have possible effects on the interferon-gamma concentration respectively. This model decomposes the total variance into between-individual error (or bias) and within-individual error (or imprecision). Herein, "between-individual error" indicates deviation in interferon-gamma values caused by the difference between Harvesters or ELISA operators, and "within-individual error" represents fluctuation of interferon-gamma values measured by a single Harvester or ELISA operator.

As shown in Table 2, mean square error indicating magnitude of within-individual error was large in all conditions of the first trial, which was indicated by remarkably large CV (> 20%) for Nil, TB-Antigen and Mitogen. Furthermore, in the condition of Mitogen, the mean-square value directing the effect of ELISA, or "between-individual error", was significantly large ($P = 0.017$). In the other two conditions, the effects of ELISA and Harvest were also considerably large but did not reach significant levels, as compared with the corresponding mean square errors. These findings indicate that their performance is unstable. Problems specific to ELISA and Harvest should be considered, although not statistically significant in all conditions.

Analysis of heterogeneous error variance in the first trial

We then analyzed which factor affected within-individual error. Because two laboratory members were involved in each step of this experiment, we assumed that within-individual error, i.e. error variance, could be different depending on the personnel in each step. Thus, we chose a fixed-effect model with heterogeneous variance of error affected by Harvest and ELISA (Table 3).

In Nil condition, difference in error variance was statistically significant between Harvesters A and B ($P = 0.0040$), when error variance caused by ELISA operator was not considered. Difference of error variance caused by ELISA operators A and B was also significant ($P = 0.024$), when error variance caused by Harvester was not taken into account. These findings imply that under the model, the error variance was affected significantly by different Harvesters or ELISA operators, respectively.

Intervention

By means of the above-mentioned statistical analysis of the first trial, we identified several points to be improved: a) there was a considerable number of outliers. Within-individual error was large and between-individual error

Table 2: Three-way analysis of variance in the first trial.

	Nil	TB-Antigen	Mitogen
Mean (IU/ml)	0.7821	5.4013	15.3638
Harvest			
Mean Square	0.0000	1.5252	51.2656
F value	0.0000	0.2200	2.5600
P value	0.9984	0.6482	0.1404
ELISA			
Mean Square	1.8838	12.3026	161.3535
F value	1.2600	1.7800	8.0700
P value	0.2847	0.2112	0.0175
Error			
Mean Square	1.4741	6.8935	19.9916
Root Mean Square	1.2142	2.6255	4.4712
Coefficient of Variation (%)	155.2476	48.6099	29.1022

Table 3: Error variance affected by Harvester (left) and error variance affected by ELISA operator (right) in the first trial.

$\varepsilon i.$	Harvester	*P* value	$\varepsilon .j$	ELISA operator	*P* value
Nil	A:1.9150 B:0.0160	0.0040	Nil	A:0.0036 B:3.2723	0.0244
TB-Antigen	A:2.9897 B:9.7114	0.2546	TB-Antigen	A: 0.1270 B:15.2216	0.0830
Mitogen	A:33.5782 B: 5.6792	0.2780	Mitogen	A:41.1154 B: 3.0221	0.3584

was also comparably large, and b) within-individual error was affected by both Harvesters and ELISA operators at least when Nil was measured.

Based on these results, an intervention was introduced: 1) reviewing all procedures of Harvest and ELISA, 2) reconsidering and strengthening standard operation procedures, 3) checking working condition of machines, and 4) developing a checklist for countercheck. First, we attempted to find out which procedure of harvesting and ELISA operation would be unstable and all questionable manipulations were listed up. Essential laboratory skills, such as mixing the solution by pipetting, were reviewed. Secondly, standard operation procedures were rechecked and corrected seeing that the laboratory personnel were handling three blood collection tubes and three other plasma storage tubes from each blood donor at a time, they should take every care to identify the tubes during Harvest and ELISA and to confirm the right position of corresponding tubes. Thorough instruction for handling ELISA plates and tubes with manipulation of the pipette was given to avoid carry-over error or contamination. After intensive discussions, more attention was paid to basic laboratory practice and reduction of preventable mistakes. Thirdly, performance of the ELISA plate washer and reader and the quality of distilled water were also checked. Technical requirements from the manufacturer, such as temperature for reagent reservation, time of incubation, were strictly followed. Finally, a checklist for the countercheck of each step was developed for practical use.

General assessment by CV before and after intervention

To assess the overall improvement after intervention, CV was compared between the two trials. Because variation due to Harvest and ELISA was of interest, CV adjusted by the effect of specimens was calculated and used. The CV had decreased remarkably in each condition of the second trial, as compared with that of the first trial, indicating the overall improvement of test performance after intervention (Table 4).

Evaluation of outliers and three-way ANOVA in the second trial

In the second trial, only one outlier was seen in Nil condition (Mahalanobis D = 2.59); the number of outliers was lower than that of the first trial.

We then proceeded to analyze the change of parameters that had possibly contributed to overall improvement of test performance. As shown in Table 5, both mean square error and mean-square values showing effects of Harvest and of ELISA were markedly lower in the second trial. The former implies the decrease in within-individual error and the latter shows the reduction of between-individual error. The latter change was also clearly shown when differences of least square means between Harvesters and between ELISA operators in each condition of the second trial were compared with those in the first trial (Figure 1).

Analysis of heterogeneous error variance affected by harvester and ELISA operator in the second trial

In contrast to the first trial, there were no significant differences of error variance affected by Harvesters or ELISA operators (Table 6). This finding showed that the heterogeneous error variance indicating personnel-dependent unstableness was small enough in each step of the second trial.

Assessment of heterogeneous variance between the two trials

We further evaluated the decrease in overall within-individual error statistically. For this purpose, we used a fixed-effect model with heterogeneous variance between the two trials. Under the assumption that influence of Harvest and ELISA was not changed between the two trials, estimated overall error variances of the two trials were com-

Table 4: CV adjusted by specimen in the two trials.

	CV (%)	
Condition	1st trial	2nd trial
Nil	150.5036	2.1661
TB-Antigen	48.6219	2.3967
Mitogen	38.1630	9.8776

Table 5: Three-way analysis of variance in the second trial.

	Nil	TB-Antigen	Mitogen
Mean (IU/ml)	0.2308	0.3071	11.0017
Harvest			
Mean Square	0.0000	0.0000	3.4225
F value	1.0000	0.1000	3.2100
P value	0.3409	0.7572	0.1036
ELISA			
Mean Square	0.0000	0.0000	0.0770
F value	1.0000	0.4000	0.0700
P value	0.3409	0.5393	0.7937
Error			
Mean Square	0.0000	0.0001	1.1707
Root Mean Square	0.0050	0.0079	1.0330
Coefficient of Variation (%)	2.1661	2.5615	9.3898

pared. As shown in Table 7, values indicating the overall within-individual error in all conditions had significantly decreased in the second trial ($P < 0.0001$).

Discussion

We have demonstrated that a study design BIBD and statistical analysis using fixed-effect models with heterogeneous variance of error are useful for objective and quantitative assessment of laboratory testing for the first time. A series of experiments in our study clearly showed that proficiency of the personnel was improved by an appropriate intervention between the first and second trials of a two-step ELISA-based immunoassay for tuberculosis newly introduced to a resource-constrained laboratory.

Design of clinical experiments including block designs can be used to estimate effect of factors and their possible interaction [10]. In block designs including BIBD, introduction of blocks usually provides extra precision for comparison of other factors, while difference between blocks is of no intrinsic interest [10]. In our proficiency testing, variation of individual specimens was not the point of interest, but analysis of the other two factors, Harvest and ELISA was of importance. Roles of laboratory members are occasionally changed because of limited manpower. In such a case, our analysis is indispensable for assessment of their individual skills in each step of the testing, since this kind of approach has not been evaluated by the conventional IQC methods [11].

Previous studies in clinical fields other than laboratory medicine showed the advantage of BIBD over the sample size [12-14]. In the present study, this design enabled us to evaluate essential components of the blood testing procedure systematically without collecting an extra specimen from each donor. If all combinations of Harvesters and ELISA operators were to be tested at the same time, a twice-larger volume of blood should be collected from each volunteer, however, obtaining consent of this often causes difficulties in a country where blood sampling is not easily accepted. We have shown furthermore that this design is suitable for clinical settings in which many different specimens are to be handled at the same time.

In the first trial before intervention, we found that within-individual error was large and between-individual error tended to be so. However, a number of outliers also affected both within- and between-individual errors. The cause of outliers was probably due to mixing up of speci-

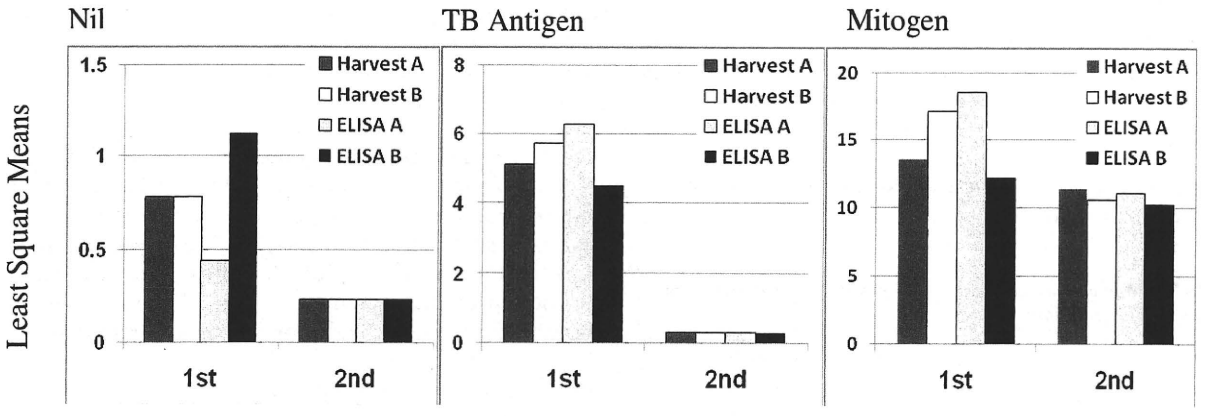

Figure 1
Least square means of measurements in the first and the second trials. Differences in least square means between Harvesters and between ELISA operators in the conditions of Nil, TB-Antigen and Mitogen in the second trial were compared with those in the first trial.

Table 6: Error variance affected by Harvester (left) and error variance affected by ELISA operator (right) in the second trial.

$\varepsilon i.$	Harvester	*P* value	$\varepsilon .j$	ELISA operator	*P* value
Nil	NE* (A > B)		Nil	A:0.000014 B:0.000035	0.4832
TB-Antigen	A:0.0001 B:0.00002	0.2391	TB-Antigen	NE (A > B)	
Mitogen	A:2.1184 B: 0.1788	0.3291	Mitogen	A:1.9065 B: 0.2747	0.33347

*NE = not estimable by this calculation.

men tubes or contamination of samples resulting from unfamiliar handling of multiple samples, although this was not easily determined [15,16]. Using a fixed-effect model with the heterogeneity of error variance, we further illustrated that within-individual error was affected by Harvesters and ELISA operators. The results indicated that there were problems with both steps of Harvest and ELISA, and with both laboratory members, and this represented a strong motivation to improve the skills of the laboratory personnel in both steps of Harvest and ELISA.

After timely intervention including checkup of all steps and standard operation procedures, marked improvement was observed in all parameters including CV, a general parameter for precision of measurements [8]. In case of IGRA in this study, CV should be kept less than 10% [17,18] and in the second trial, this criterion was met satisfactorily.

We propose as a consequence the following action plan to improve diagnostic capacity in resource-constrained settings. This could be generalized not only for complicated immunoassay for infectious disease but also for other kinds of clinical tests:

- Set the target CV derived from simple one-way ANOVA model of specimen (for example, 10%). This value should be defined before the commencement of study.

- Design experiment to evaluate between- and within-individual error.

Table 7: Difference in estimated overall within-individual error between the two trials.

	Estimated overall error variance (ε_{ijk})	
Condition	1st trial	2nd trial*
Nil	1.3866	0.000025
TB-Antigen	6.9003	0.000062
Mitogen	35.3152	1.0814

*P < 0.0001

- Conduct experiment.

- Analyze data with ANOVA model with and without heterogeneous error variance.

- If CV exceeds the target, review the operating procedures.

- Conduct experiment a second time.

- Consequently analyze data to ascertain any improvement.

- Return to step 5 until CV becomes less than the target.

In-house quality control for effective transfer of skills is a topic of interest in our proposal and this should be carried out easily, at a low cost, whilst assuring objective and quantitative assessment in a clinical laboratory where resources such as reagents, manpower and feasibility of sample collection are limited. Our plan meets the above requirement. Measurements could be sent via the internet and analyzed in a statistical way by a joint-research facility inside or outside the country and an immediate feedback should be sent in an appropriate manner. Such continuous efforts to share information are important to maintain quality levels over a long distance [19].

In this age of evidence-based medicine and development of new diagnostic technologies, quality of laboratory tests is essential. There is an urgent need for validation and standardization of the new assays before they are adopted into clinical diagnostics [20]. Until such time as an effective quality control system is established, our approach is valuable to assure the quality of laboratory tests for timely diagnosis and treatment of infectious diseases. Another favorable design or analytical method might be suggested by others in the future studies, seeing that no standard way of quality monitoring has been proposed so far. We expect that the successful experience gathered in the present study will provide useful information for further comparison and discussion.

Our study has some limitations. It was obvious that outliers influenced statistical analysis in the first trial and exact causes of error in each condition were not clearly specified by the present analysis itself [15]. Through repeated experiments, the causes of error might be clearer, although all errors in our study decreased dramatically after a single intervention. We should also emphasize in conclusion, that a number of procedures should be combined to establish a total quality assurance system.

Conclusion

In a setting where a modern quality control system has not been entirely established, a laboratory test could be assessed quantitatively and such objective assessment is helpful for quality improvement of the test, if an appropriate design of experiment and statistical method are chosen. The design of experiment BIBD and analytical models for ANOVA were useful for objective assessment of individual skills in each stage of a multi-step immunoassay for tuberculosis in a laboratory with limited resources. A proposed plan to assess the level of proficiency might be useful for skill improvement of clinical testing especially for infectious diseases when monitoring is difficult to assure the sustainability of the technology transferred.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NTLH participated in supervising the on-site implementation of the study, drafting the paper or substantially revising it. NI was responsible for making conception, design and overall supervision of the study, analysis and interpretation of data, drafting the paper or substantially revising it. NK participated in making conception and design of the study, analysis and interpretation of data, drafting the paper or substantially revising it. LTH carried out the immunoassays. DBT participated in on-site implementation. VTXT carried out the immunoassays. IM participated in technical transfer and supervision. NH was responsible for technical transfer and supervision. KH was responsible for technical transfer and supervision. SS participated in conception and design of the study. LTL participated in conception, design and supervision of the study. All authors read and approved the manuscript.

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Overview: "Children's Toxicology", a renovating study field of irreversible "early exposure-delayed effects"

Jun Kanno

Division of Cellular and Molecular Toxicology, Biological Safety Research Center, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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ABSTRACT — "Children are not small adults". This is a well-known phrase, especially in the clinics for diagnosis, efficacy of treatment, side effect, and prognosis. However, in the field of toxicology, this issue has long been a challenge. The knowledge has been limited to the differences in metabolism and other physiological factors. Currently available test guidelines for fetuses and immature animals are teratogenicity and reproductive toxicity studies. These tests look for straight-forward (essentially macroscopic) outcomes established within a rather short period of exposure to the test substances. However, recent advances in molecular toxicology allow combination of *in vitro* and *in vivo* studies at molecular levels. The target molecules and receptors can be identified in quantitative fashion and at the fine structure levels around and below the resolution of normal light microscopy. Such expansion of the knowledge lead us to consider a rather new category of "receptor mediated toxicity" or "signal toxicity". Such non-organic insults would merely induce transient effects on adults. However, there are growing evidences that such slight insults on the developing and maturating organisms can leave irreversible effects that become overt in adulthood. As an overview, toxicology has entered a new phase where children's toxicology becomes a renovating study field of the irreversible "early exposure-delayed effects".

Key words: Children's toxicology, Receptor-mediated toxicity, Signal toxicity, Early exposure-delayed effect

INTRODUCTION

Toxicology is a study to analyze interaction between living organisms and xenobiotics, and its final goal is to secure the safety of humans and environment in modern life where various products and technologies are used. Up to now, the majority of toxicological tests to evaluate the toxicity of a particular substance are utilizing experimental animals as a surrogate of humans. The results obtained from such animal tests are extrapolated to humans for the settlement of various kinds of regulation on the test substances, i.e. food additives, pesticides, industrial chemicals, medicines, etc. In cases of pharmaceutical products, clinical trials (human tests) are available. However, these are rather exceptional occasions for toxicology as a whole. It would be very difficult for non-pharmaceutical objects to test on humans, and even for pharmaceuticals, human trial for children including fetuses have many difficulties.

Current toxicological testing protocols are based on an assumption that both experimental animals and humans share common basic structure of the body and thus similar biological reaction. Most of those toxicological studies are based on "diagnosis" of the symptoms of experimental animals in a similar fashion to give a diagnosis to human patients. Because the fine structure and function of the bodies are still unknown, both humans and animal bodies are "black boxes" responding to the test substances by showing various symptoms. Usually, the "no observed adverse effect level" (NOAEL) or "no observed effect level" (NOEL) is given by such tests. Since the basic nature of species differences and individual differences are not known, a number called "safety factor" was invented to extrapolate animal NOAEL/NOEL data to humans (Benford, 2000). Normally, a factor of 10 for the species and another 10 for individual differences, thus 100 as a whole, is used to set lower NOAEL/NOEL levels for humans. This approach has been working well for the majority of test substances. Not surprisingly, however, there are some exceptions. Thalidomide is a best-known example (Newman, 1985). Phocomelia, a spectrum of malformation of limbs, was induced in offspring of tha-

Correspondence: Jun Kanno (E-mail: kanno@nihs.go.jp)

lidomide-treated pregnant women, but not observed in offspring of mice and rats. Therefore, more precise toxicity evaluation/prediction is obviously needed for safer assessment. An approach that enables us to point out molecular mechanisms of toxicity would be essential for such needs and to better understand the species-specific responses.

DISCUSSION

To modernize the toxicology and improve the accuracy of safety assessments, we are attempting to describe and understand the organism-xenobiotics interaction at the molecular levels. Different for other exploratory studies, a major prerequisite is that the Toxicology must be prepared for any unexpected or unpredictable responses. Thus, the approach must be comprehensive. Consequently, we adopted a whole-genome cDNA microarray system for a comprehensive monitoring of the transcriptome, and launched the Percellome Toxicogenomics Project, of which the ultimate goal is to illustrate out the whole regulatory pathways induced by xenobiotics in the experimental animals, mainly mice, including embryo (Kanno *et al.*, 2006).

On top of that, there is an important factor of toxicology, that it the "time frame" such as acute, chronic and delayed toxicity. Among them, researches for the assessment of delayed toxicity targeted for children (including fetus and infants) is becoming very important. It is very likely that the children have a chance to be exposed in daily life to a series of substances which can be a cause of delayed toxicity, especially, of the highly evolved systems, that is endocrine, immune and central nervous system. Such chemical substances can affect the developing systems at a dosage lower than the dosage that induces overt cytocidal changes that would link to immediate appearance of symptoms. For example, our recent experience on the perinatal exposure study (Tanemura *et al.*, 2009) which resulted in the emergence of delayed effects on neurobehavioral endpoints can be explained by a metaphor. That is, "No one turns on power when building a computer, but the living brains are built under the "power-on" situation". It is very likely that the developing brain needs proper or normal signals to build up its fine structures and functional networks (Cohen-Cory, 2002). At this stage, if the signals are disrupted by exogenous insults, it may result in malformation of the fine structure of the brain system. In this case, it is not necessary to directly kill the nerve cells during exposure. The malformation of fine structure/functional network will become symptomatic when the animals grow up to adults. On the other hand, most of those insults to adults would end up in reversible and transient changes.

Such delayed toxicity cannot be readily detected by currently available functional observational battery-(FOB-) based neuronal test system. Our new findings fall into the category of "early exposure- delayed effect". As mentioned above, nervous systems of developing organisms are susceptible to signal disruption which could lead to the delayed neurobehavioral anomaly. Toxicology is asked to prepare to respond to such new types of toxicity or "signal toxicity" with a consideration on the mechanisms which could explain the severity and irreversibility specific to children.

In conclusion, the 35th Annual Meeting of the Japanese Society of Toxicology had raised "Children's Toxicology" as one of its main Themes, and organized Special lectures, five Symposiums and two Workshops on Children's Toxicology of various targets and pending problems, which includes central nervous system, immune system, and endocrine system as targets, as well as problems in pharmacology i.e. issues on children's preclinical and clinical trials and on the off-label use of drugs. This special issue of the Journal of Toxicological Sciences gathers the peer-reviewed papers presented by the authors who participated in the lectures/symposiums/workshops on Children's Toxicology at the meeting.

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